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Effects of Lactic Acid produced by lactic acid bacteria on Prodigiosin Production from *Streptomyces coelicolor*

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Abstract

Background: Several approaches were carried out ranging from improving nutrient sources to metabolic engineering of *Streptomyces coelicolor* or co-cultivation with cell-free supernatant from lactic acid bacteria.

Methods: Lactic acid isolated from *Leuconostoc mesenteroides* and *Lactobacillus plantarum* was detected on thin layer chromatography and quantitated by spectrophotometry and then, added into medium that was used to check for prodigiosin production ability of *Streptomyces coelicolor*. The intracellular and extracellular prodigiosin amounts were measured by spectrophotometry.

Results: Approximately 0.109 g/mL - 0.117 g/mL of lactic acid produced by *Lactobacillus plantarum* and *Leuconostoc mesenteroides* (10^6 CFU/mL) was obtained. The production of intracellular prodigiosin treated with different extracts from lactic acid bacteria after 6 days of incubation was less than nine fold when compared with the culture without lactic acid. The production of extracellular prodigiosin treated with different extracts from *L. mesenteroides* lactic acid bacteria after 6 days of incubation was higher than *L. plantarum*, but less than the culture without lactic acid.

Conclusion: There was inhibition effect of lactic acid produced by *Lactobacillus plantarum* and *Leuconostoc mesenteroides* on intracellular and extracellular prodigiosin.

Keywords: Prodigiosin, *Streptomyces coelicolor*, lactic acid bacteria, lactic acid, *Leuconostoc mesenteroides*, *Lactobacillus plantarum*

Introduction

Prodigiosins, the bioactive secondary metabolites produced at later growth stages of several bacteria (both Gram-positive and Gram-negative microorganisms), have gained interest of many researchers since first isolated from *Serratia marcescens* due to their promising therapeutic and commercial benefits [1, 2]. The bacterial compounds, characterized by a common pyrrolyl-dipyrrolyl-methene skeleton, were distinguished by their natural striking red color and historically well known for “bleeding red” reports. Prodigiosins have a wide range of therapeutic applications, including antimalarial, antibacterial, immunosuppressive and most notably anticancer [3-7]. *In vivo* studies reported prodigigiosin induced apoptotic activity against a group of several cancer cell lines, such as colon, gastric, kidney, lung and breast cancers with low cytotoxicity to non-cancerous cells [8, 9]. Aside from biological activities, the red pigment also displays a potential role in industrial applications as a natural food colorant and textile material [10, 11]. These properties mark the red pigmented compounds as a powerful research candidate.

Among the prodigiosin-producing *Actinomyces*, studies have focused on *Streptomyces coelicolor A3(2) (S. coelicolor A3(2))* strain, a gram-positive, spore-forming soil bacterium, owing to biosafety reasons and its ability to synthesize both linear side chain member undecylprodigiosin and a cyclic side chain member, *streptorubin B* in 2:1 ratio [12]. Several approaches on

inducing the red-pigmented secondary metabolites of *S. coelicolor* were carried out over recent years. A study in 2011 by Luti and Mavituna found pigment production could be enhanced by dead cells from *Bacillus subtilis* and *Staphylococcus auerus* [13]. Another report in 2011 suggested the use of *Verticillium dahliae* and *Arabidopsis thaliana* roots increased the production of undecylprodigiosin [14]. These studies raised a new interesting strategy in improvement of prodigiosin biosynthesis with bacterial dead cells. Since lactic acid bacteria (LAB) are considered non-pathogenic while also produce various bioactive compounds, several strategies have developed to explore the use of these probiotics on the biosynthesis of prodigiosin. A more recent report in 2014 by Tu Nguyen *et al* recorded a 6-fold upregulation of red-pigmented product using cell-free supernatant extracted from heat-killed *Lactobacillus rhamnosus* [15]. However, the bioactive compounds associated with the synthesis of this secondary metabolite remained unidentified. Some suggestions involved the presence of N-acetyl glucosamine which was assumed to trigger the antibiotic production in *Streptomyces*, or a membrane product that might play a role as an elicitor in case of red pigment production.

Following the new approach, this paper investigated on the effect of lactic acid, a bioactive compound extracted from cell-free supernatants of *Leuconostoc mesenteroides (L. mesenteroides)* and *Lactobacillus plantarum (L. plantarum)* on red pigment biosynthesis from *S. coelicolor* to contribute to further understanding of the effect of LAB on prodigiosin production from *S. coelicolor*.

Materials and methods

Bacterial strains and growth culture

Inoculum (10^6 CFU/mL) of *L. mesenteroides* and *L. plantarum* were cultured in 50 mL of De Man-Rogosa-Sharpe (MRS) medium (Himedia, India) in 48 hours at room temperature before harvesting. The collected cultures were heated for 1 hour at 70°C and centrifuged (10 000 rpm, 25°C) to obtain cell-free supernatants.

Lactic acid preparation

The isolation of lactic acid was performed in several steps according to previous study [16]. Briefly, after 48 hours of incubating, cultures of LAB were alkalized with $Ca(OH)_2$ to allow crystallization of calcium lactate before acidified with 63% H_2SO_4 to pH 1.8 and centrifuged in 30 minutes at 10 000 rpm to remove debris and collect lactic acid.

Detection of lactic acid by Thin-Layer Chromatography

To detect lactic acid in LAB, the silica gel-coated TLC plates (G F254, Merck) were used as stationary phase with n-butanol: acid formic (95:5) (Fried, 1999) [17] as the mobile phase. The standard solution was DL-lactic acid (Sigma). Observation of TLC result was performed under 254 nm UV light.

Determination of lactic acid concentration

Lactic acid produced by LAB was quantified using spectrophotometric method developed by Borshchevskaya in 2016 [18]. An aliquot of 50 µL of solution containing lactic acid was added to 2 ml of 0.2% $FeCl_3$ and stirred well before measuring at A_{390} for absorbance value. A series of known concentrations (0-0.3%) of standard DL-lactic acid (85% in water, ρ = 1.209 g/mL, Sigma) was used to construct a calibration curve. Results were expressed in g/mL unit.

Preparation for prodigiosin production

Inoculation of *S. coelicolor* was carried out using spore stock in 20 mL of Yeast Extract - Malt Extract (YEME) medium. The culture was incubated at 28°C for 48 hours in shaking condition. 500 µL of solution containing 500 µg of lactic acid at pH 7 obtained from LAB was further added seperatedly to the shaken flasks to observe color change in total 6 days. Final concentration of prodigiosin was measured with photometric analysis.

Photometric analysis of prodigiosin concentration

To determine the extracellular value of prodigiosin production, centrifugation was carried out to collect the cell-free supernatants. After that, the obtained solutions were adjusted to pH 2 before analysing at A_{530} using spectrophotometer [19]. For intracellular pigment production, cell pellets were applied onto a paper filter and washed twice with 20 mL of distilled water. After rinsing 3 times with 30 mL of acetone, the fraction was further evaporated to dryness and resolved in methanol before measuring at A_{530} for prodigiosin value.

The concentration of red pigment was calculated in mg/L using following equation:

$$c\left[\frac{mg}{L}\right] = \left(x \times \frac{M}{\varepsilon}\right) \times 1000$$

Where c = concentration, x = measured absorbance value, M = molar mass which is 393.5649 g/mol for prodigiosin and ε = molar extinction coefficient of prodigiosin (100,150 $M^{-1}cm^{-1}$).

The analysis results were performed with one-way ANOVA on SPSS 20, taking $p < 0.05$ as the lowest limit of significance.

Results and discussion

Lactic acid qualification and quantification

Dectection of lactic acid under UV light was represented in Fig. 1. Lactic acid extracted from both strain of LAB expressed the same Rf value as the standard lactic acid (85% in water, Sigma), indicated the presence of lactic acid in the isolated samples.

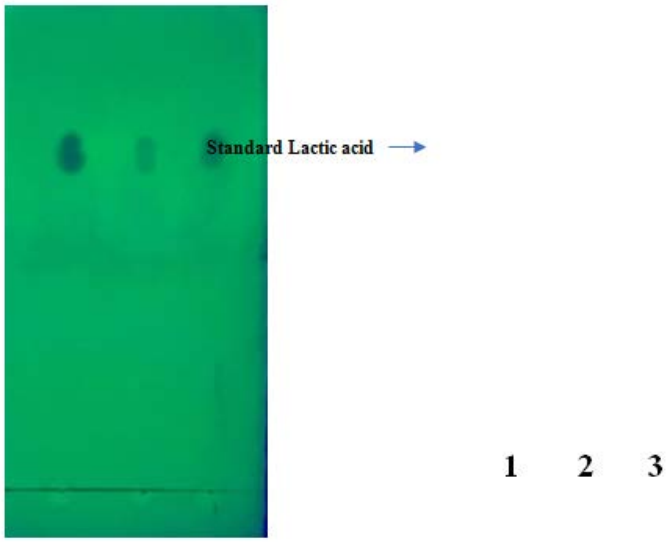

Fig. 1: Lactic acid detection on Thin-layer Chromatography. Standard lactic acid (1); *Leuconostoc mesenteroides* (2); *Lactobacillus plantarum* (3)

Lactic acid quantification from LAB was depicted in Table 1. Approximately 0.117 g/mL of lactic acid produced by *L. mesenteroides* was calculated insignificantly to that of *L. plantarum*, which contained only 0.109 g/mL.

Table 1: Determination of lactic acid concentration produced by *Leuconostoc mesenteroides* and *Lactobacillus plantarum*

Lactic acid bacteria	Lactic acid concentration (g/mL)
Leuconostoc mesenteroides	0.117 ± 0.005
Lactobacillus plantarum	0.109 ± 0.006

The experiment was triplicated with results are reported as means ± S.D.

Effects of lactic acid at different pH level on the growth of *Streptomyces coelicolor*

In order to study the effect of lactic acid on the pigment production of *S. coelicolor*, the growth of *Streptomyces* was checked with lactic acid at pH 6, 7, 8 and the CFU/mL was illustrated in Fig. 2. Since pH of culture medium has been reported to play an important role in pigment biosynthesis, as it might affect the function of several proteins involved in the metabolism of amino acids required for prodigiosin production [20], optimizing the pH of lactic acid was essential to achive the highest yield of secondary metabolites.

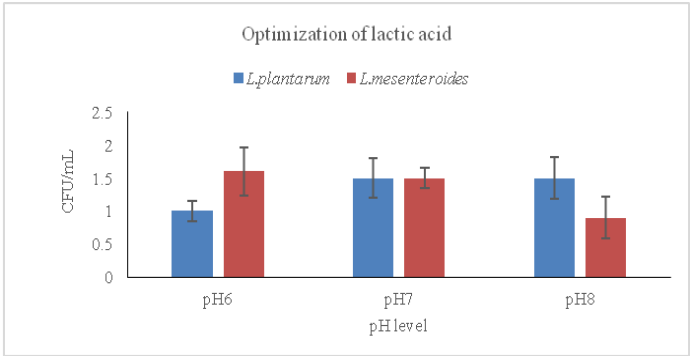

Fig. 2: Effect of lactic acid on the growth of *Streptomyces* in different pH level

From Fig. 2, *S. coelicolor* could grow well in pH 6 and 7. Therefore, pH 7 was optimal for study on prodigiosin production of *S. coelicolor* in case of medium supplied with lactic acid prepared from lactic acid bacteria.

Effects of lactic acid on prodigiosin production

A reddish color developed in untreated control flask indicated the production of secondary metabolites from *S.*

coelicolor. After 3 days of observation, a slight change in color was shown in *L. mesenteroides* group, shifted toward red spectrum. With lactic acid extracted from *L. plantarum*, no difference in color could be seen in at the end of day 3 and the medium color remained unchanged after 6 days of incubation (Fig. 3). This might suggest an inhibition effect of carbon source as lactic acid on the production of red pigment in *S. coelicolor*. Aside from color change, the pH of culture increased after incubation with lactic acid, which was estimated that lactic acid might converse any compounds in *S. coelicolor* cultures causing pH changing and suppression of pigment production (Fig. 4).

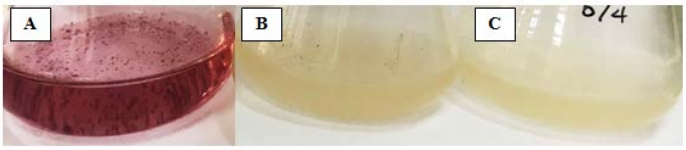

Fig. 3: Prodiogiosin production without lactic acid (A) and lactic acid-treated group, *L. mesenteroides* (B) and *L. plantarum* (C).

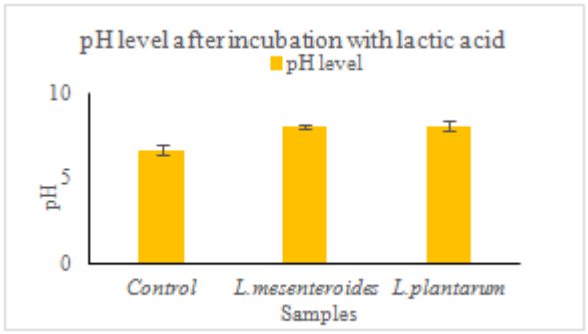

Fig. 4: pH level after 6 days of incubation with lactic acid from *L. mesenteroides* and *L. plantarum* compared with control group

Table 2: Production of intracellular prodigiosin treated with different extracts from lactic acid bacteria after 6 days of incubation

Extracts adding to culture	Intracellular prodigiosin production (mg/L)
Control	0.370 ± 0.23
L. mesenteroides	0.044 ± 0.04
L. plantarum	0.016 ± 0

Results are mean value of triplicate measurements ± S.D. The control group was culture of *S. coelicolor* without the addition of lactic acid

The afterward values from photometric analysis revealed intracellular pigment yielded from both treatments decreased intensively with 8 to 23-folds drop in concentration as compared to that of untreated control group (Table 2). Interestingly, lactic acid produced by *L. plantarum* expressed a higher down-regulating effect than the same compounds extracted from *L. mesenteroides*, as the former LAB reduced the red pigment production to approximately 0.016 mg/L, 28-folds lower than the yield from *L. mesenteroides* lactic acid group. The low production of intracellular prodigiosin resulted from both treatments suggested a hypothesis that this compound might act as an inhibitor to the synthesis of secondary metabolite from *S. coelicolor*.

The extracellular prodigiosin concentration recorded in Table 3 further strengthened the above statement, with lactic acid produced by *L. plantarum* continued to display a strong inhibitory effect on red pigment biosynthesis. The treatment with bioactive compound from *L. plantarum* exhibited a yield of only 0.07 mg/L in contrast to the 0.48 mg/L concentration in control. Extracellular pigment product from *L. mesenteroides* lactic acid group surprisingly raised, reached as high as 0.323 mg/L although the amount of lactic acid added in cultures was not different (around 500 – 600 µg). Probably, there was different isoform of lactic acid produced by two bacteria. More study should be done to clarify the isoforms of lactic acid produced by different bacteria.

Table 3: Production of extracellular prodigiosin treated with different extracts from lactic acid bacteria after 6 days of incubation

Extracts adding to culture	**Extracellular prodigiosin production (mg/L)**

Control	0.48 ± 0.39
***L. mesenteroides* lactic acid**	0.323 ± 0.25
***L. plantarum* lactic acid**	0.070 ± 0.07

Results are mean value of triplicate measurements ± S.D.

Conclusion

As the poor production of both intracellular and extracellular red pigment from lactic acid-treated group was depicted by spectrophotometric assay, it could be assumed that lactic acid from *L. plantarum* inhibited prodigiosin synthesis in *S. coelicolor* while *L. mesenteroides* only exhibited the down-regulation effect on intracellular prodigiosin. More study should be done to uncover the mechanism of lactic acid on prodigiosin production in different sources of prodigiosin.

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